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Computational studies of transport in ion channels using metadynamics

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Abstract

Molecular dynamics simulations have played a fundamental role in numerous fields of science by providing insights into the structure and dynamics of complex systems at the atomistic level. However, exhaustive sampling by standard molecular dynamics is in most cases computationally prohibitive, and the time scales accessible remain significantly shorter than many biological processes of interest. In particular, in the study of ion channels, realistic models to describe permeation and gating require accounting for large numbers of particles and accurate interaction potentials, which severely limits the length of the simulations. To overcome such limitations, several advanced methods have been proposed among which is metadynamics. In this algorithm, an external bias potential to accelerate sampling along selected collective variables is introduced. This bias potential discourages visiting regions of the configurational space already explored. In addition, the bias potential provides an estimate of the free energy as a function of the collective variables chosen once the simulation has converged. In this review, recent contributions of metadynamics to the field of ion channels are discussed, including how metadynamics has been used to search for transition states, predict permeation pathways, treat conformational flexibility that underlies the coupling between gating and permeation, or compute free energy of permeation profiles.

Keywords: membrane proteins; free energy; K^+ channels; Na^+ channels; enhanced sampling; molecular dynamics; simulations

Abbreviations

MD: Molecular Dynamics

CV: Collective Variable

WTMetaD: Well-Tempered MetaDynamics

BE-META: Bias-exchange Metadynamics

US: Umbrella Sampling

Introduction

Ion channels regulate the flow of ions across the cell membrane. They are ubiquitous to all life kingdoms. The human genome encodes for more than 400 different ion channels, representing around 2% of all known genes, but ion channels are also essential in lower organisms where they are involved in infectious diseases and act as toxins or antimicrobial agents. The two main functional characteristics of these membrane proteins, that are usually employed to classify them, are selectivity and gating. Selectivity is the capacity of ion channels to discern among different ionic species, favoring some particular ions that can permeate the pore and excluding others from conduction events. Gating is the ability of these proteins to switch between conductive and non-conductive states in response to physico-chemical stimuli. Cells are equipped with an arsenal of ion channels, characterized by different selectivity and gating attributes. This heterogeneity in the population of channels is essential for a variety of biological functions, such as nerve transmission, muscular contraction, and environmental sensing. Consequently, any theoretical model designed to reproduce these biological processes ought to describe selectivity and gating in quantitative rather than just qualitative terms. Indeed, a solid description of selectivity and gating was the main feature of the celebrated Hodgkin and Huxley model of the action potential [1]. With the advent of experimental atomic structures of membrane proteins [2] from X-Ray crystallography, NMR and more recently cryo-electron microscopy, compelling insights into the physical processes responsible for selectivity and gating in ion channels have emerged. In combination with these experimental structures, computational tools and theoretical methods have been used to simulate the dynamical behavior of complex biological molecules, such as ion channels, at the atomic level. Molecular Dynamics (MD) simulation is certainly the most popular technique for simulating the dynamics of complex biological systems. In MD simulations, atomic forces are described by empirical functional forms and sets of parameters. To date, force fields have largely been based on non-polarizable, additive functions, where the partial atomic charges of the system are fixed and account for induced electronic polarization in a mean-field manner. These crude simplifications in the description of the inter-atomic forces in classical force fields are a major shortcoming for the description of ion channels by atomistic simulations [3]. However, work on improved

force fields that include explicit polarization has been reported in numerous studies [4-7]. A further complication is the time scale accessible in MD simulations of ion channels. In this respect, diverse approaches have been proposed to enhance sampling and to extend the time scales of the processes accessible by atomistic simulations. These enhanced sampling techniques, including metadynamics and related approaches, have been successfully applied to investigate complex mechanisms of ion permeation and gating in ion channels. In this review, we highlight the recent contributions of metadynamics to the field of ion channels, and give examples of how this algorithm has been used to search for transition states, predict permeation pathways, treat conformational flexibility that underlies the coupling between gating and permeation, and compute free energy profiles of permeation or gating.

Simulation and modeling of ion channels

The most straightforward way to simulate atomistically selectivity, permeation and gating in ion channels is to replicate an electrophysiological experiment *in-silico*. In order to reproduce an electrophysiological experiment using computer simulations it is necessary to control the boundary conditions for ion concentrations and membrane potential. A simple strategy to mimic the presence of a membrane potential in an MD simulation is to apply a constant electric field in the direction orthogonal to the lipid membrane. This approach has been used to study conduction events in different types of ion channels, including K^+ channels [8, 9] and Na^+ channels [10]. The control of boundary conditions for ion concentrations requires dedicated algorithms. Indeed, as MD simulations of membrane proteins are performed using periodic boundary conditions, the ionic solutions at both sides of the lipid bilayer are part of the same compartment, and thus, ion concentrations are necessarily the same at the equilibrium. A possible strategy to establish concentration gradients between the two sides of the membrane is to bias the ion movements using some external driving forces [11]. If ions of one type are driven from one side of the membrane to the opposite side, they will spend on average more time on the compartment where they are pumped into, which in turn will give rise to a concentration gradient for that particular type of ions. An alternative strategy to control ion concentrations is to include in the simulation domain two parallel lipid membranes. The presence of two membranes divides the simulation domain in two compartments: i)

the region between the membranes, and ii) the outer space connected through the boundary conditions imposed. By creating and annihilating particles, it is then possible to maintain different concentrations of ions in these two disconnected reservoirs [12, 13]. The same approach can be followed to create an imbalance of electrical charges between the two sides of each membrane, and consequently, to control the membrane potential. The computational costs of an atomistic simulation of a double-bilayer are obviously considerable. However, the expense is partially compensated if two independent ion channels are inserted in each of the two parallel-oriented lipid membranes as this set up renders more statistics. At present, this is the simulation strategy that more closely resembles the conditions of a real electrophysiological experiment.

A strategy based on direct simulation of electrophysiological experiments *in-silico* has been used to analyze conduction events in channels with high or medium conductance (> 10 pS), and in the presence of high membrane potentials (> 100 mV) [8, 10, 14, 15]. However, many ion channels are characterized by low values of conductance (1-10 pS), and at physiological conditions, they operate in the presence of low driving forces. A robust statistical estimate of the conductance for these ion channels requires atomistic trajectories in the millisecond regime. Although MD simulations have greatly contributed to understanding the dynamics of ion channel permeation and gating, simulations are still limited by the time scales that can be routinely sampled. At present, simulation of trajectories of up to a few microseconds are feasible owing to special-purpose machines like Anton [16], the porting of MD codes to GPUs [17], and the evolution of parallel codes [18, 19]. However, we are far from being able to use these strategies routinely to study ion channels. Direct MD simulations might be capable of reproducing the conduction properties of an ion channel when the channel is stable in one particular open conformation. However, as a result of gating, ion channels switch among different functional states, and the time constants of these state transitions span a wide range of scales, from flickering events in the sub-millisecond timescale, to slow-inactivation processes that might take seconds to complete. In order to characterize the dynamics and function of an ion channel, all of its relevant states must be sampled during the simulation. Unfortunately, at present, it is not possible to reproduce these events by straightforward all atom MD simulations, and this situation is not likely to change in the near future. This

well-recognized limitation of MD has led to the development of many innovative algorithms to enhance the sampling of rare events associated with crossing high-free-energy barriers such as ion permeation and gating [20, 21]. Among such schemes are umbrella sampling, thermodynamic integration, replica exchange, adaptive biasing force or metadynamics [22].These approaches require choosing an appropriate collective variable (CV) which describes the progress of the conformational transition. Identifying a suitable set of CVs might be extremely complicated, and computationally expensive, as it will be described in the next section. However, once this set of parameters is established, the free energy profile along these CVs can be fully described as well as the equilibrium properties of the system. This approach does not provide a direct link with any dynamical behavior, which hampers the comparison between simulations and electrophysiological experiments. Nonetheless, it is possible to estimate the conduction properties by using the computed free energy profiles as input in mesoscopic models of ion conduction. This strategy was already used to estimate the conductance of different ion channels [23-25], and the results were in satisfactory agreement with experimental observations.

The main asset of a simulation strategy based on accelerated sampling and free energy calculations is that it allows investigation of processes that are not easily accessible by direct MD simulations. As mentioned earlier, many state transitions of membrane proteins are far slower than the time scales currently available for straightforward all atom MD simulations. Thus, algorithms to accelerate sampling are necessary to analyze for example the gating properties of an ion channel. Algorithms to accelerate sampling are also useful to analyze more efficiently conduction events in low conductance ion channels or permeation events of ions with low permeability, e.g. the blockage of conduction by Na^+ or Rb^+ ions in potassium selective ion channels. Moreover, recent experimental evidence suggests that the distinction between ion channels and transporters might not be as simple as initially proposed, and that for some membrane proteins, ion conduction is highly correlated with the transition states of the protein [26]. Application of enhanced sampling techniques might also proof useful to resolve this controversy.

The focus of this review is on metadynamics, an enhanced sampling technique. In the next section, a brief overview of the original algorithm is given along with some of its variants. Subsequently, metadynamics simulations of ion conduction and state transitions

in ion channels are reviewed, with a focus on the strengths and shortcomings of the technique in comparison with other available approaches.

The Metadynamics algorithm

Metadynamics, also often called the hill method, aims at enhancing rare events and reconstructing the underlying free energy landscape as a function of a set of order parameters, usually referred to as collective variables, CVs. This approach has several characteristics that have proven useful for the study of ion channels. Metadynamics can be used to accelerate state transitions along a predefined set of CVs, and at the same time render the free energy profile along them [27]. The widely spread use of this approach is due, in part, to the ease of implementation of the algorithm. The basic principles of metadynamics simulations will be described next, although readers interested in the theoretical details of the algorithm are referred to specific reviews on the topic [28].

A prior hypothesis to any metadynamics simulation is that the process under investigation can be described using a set of CVs, $S(x) = \left(S_1(x), S_2(x), \ldots, S_D(x)\right)$, where x is the microscopic state of the system. In principle, any number of CVs could be used, and the choice of this set of CVs is at the heart of a successful configurational sampling and free energy reconstruction. One of the main practical difficulties originates from the compromise between keeping the number of CVs at a minimum but at the same time exploring efficiently the multidimensional free-energy landscape, and biasing all the slow motions of the process of interest. In practice, the number of CVs rarely exceeds two or three, because the time required exploring the configurational space increases exponentially with the dimensionality of the set of CVs. Choosing the wrong set of CVs will result in incomplete or absurd results and free energies characterized by artifacts or missing features.

The state transitions of the system along the CVs are accelerated by a time-dependent biasing potential, $V_G(S,t)$. This biasing potential is updated with period τ_G by adding a Gaussian function in practice (or any other type of function in theory) centered at the current value of the CVs. Therefore, after a time interval equal to $N\tau_G$, the biasing potential is:

$$V_G(S, N\tau_G) = \omega \sum_{n=0}^{N} \exp\left(- \sum_{d=1}^{D} \frac{\left(S_j - s_d^n\right)}{\delta s_d^2} \right) \qquad \text{Equation (1)}$$

where s^n is the value of S at time step $n\tau_G$, and ω and δs are the height and the width of the Gaussian function respectively. The Gaussian terms in Equation (1) raise the value of the energy of the configurations previously visited in the course of the metadynamics run. Therefore, the biasing potential, $V_G(S,t)$, forces the system to examine unexplored regions of the phase-space, and it prevents the system from being trapped in local free energy minima (Figure 1). Such a procedure recursively flattens the free energy barriers that separate distinct thermodynamic states of interest.

A pictorial representation commonly employed to illustrate the effect of the bias is by making an analogy to a set of basins that will be filled with sand. Once enough sand (Gaussian functions) is added, the well that corresponds to the local energy basin becomes flat, and the system is free to explore other wells. What differentiates metadynamics from comparable preexisting methods is that if one keeps memory of all the positions in which the sand (the Gaussians) was deposited, a negative image of the underlying well could be reconstructed. The sand added to the system might be interpreted as a cast of the energy landscape, or in other words, the biasing potential $V_G(S,t)$ provides an unbiased estimate of the free energy profile along the CVs. The concurrence between the biasing potential and the free energy was first postulated on an empirical basis and verified empirically in several complex systems. Subsequently, it was demonstrated rigorously for systems evolving under the action of Langevin dynamics [29].

The accuracy and the computational efficiency of metadynamics simulations are affected by two parameters: the width (δs) of the Gaussian functions and the ratio between the height of the energy terms and the period used to update the biasing potential (ω/τ_G) [30]. The width of the Gaussian functions defines the space-resolution of the estimated energy profile, as it will not be possible to solve energetic features that are closer than δs. This parameter is usually defined considering the average oscillations exhibited by the CV in a classical MD simulation. The ω/τ_G ratio is a major determinant of the accuracy of the simulation. The error on the estimated energy profile is proportional to $\sqrt{w/t_G}$, which

advocates for low-energy Gaussians deposited with low frequency. Opposite conditions are required to speed up the exploration of the configurational space, as energy basins would be more rapidly 'filled' by high energy-terms added at high frequency. As a consequence, the value of ω/τ_G is the result of a trade-off between accuracy and computational efficiency. Together with the three parameters characterizing the algorithm (δs, ω and τ_G), the length of the trajectory is another critical factor; it needs to be long enough to allow the system to explore any relevant energy basins, and reach a diffusive regime in the phase-space of the CVs. However, prolonged simulations might drive the system toward high-energy states that are not physically meaningful. Moreover, the biasing potential does not converge asymptotically to the free energy profile, which further complicates the decision of when to end a metadynamics run. Once the biasing potential compensates for the free energy and the system starts diffusing along the CVs, the estimated energy profile oscillates around the actual free energy of the system. In order to get rid of these oscillations, the estimate of the free energy, $\tilde{F}(S)$, is usually calculated as the average of the biasing potential:

$$\tilde{F}(S) = -\frac{1}{t_{tot} - t_f}\int_{t_f}^{t_{tot}} V_G(S,t)\,dt \qquad \text{Equation (2)}$$

where t_f is a time step when the system has already reached the diffusive regime in the phase-space of the CVs, and t_{tot} is the total simulation time. While there is not a simple way to improve the efficiency of metadynamics simulations, after more than a decade of accumulated experience, hints are available in the literature.

An alternative form to the metadynamics algorithm, known as well-tempered metadynamics (WTmetaD), theoretically solves the problem of estimating a converged energy profile [31]. In WTmetaD, the height of the Gaussian energy terms is an exponentially decreasing function of the biasing potential. In practice, whenever an energy basin is reached, high energy Gaussians are initially used to accelerate sampling, followed by deposition of low energy ones. In this way, the estimate of the free energy obtained by WTMetaD asymptotically converges to the free energy of the system.

The main determinant of efficiency and accuracy in a metadynamics or WTmetaD simulation, like in any simulation that accelerates sampling along a set of degrees of

freedom, is the set of CVs. The ideal set of CVs needs to discriminate between initial, final, and intermediate states of the process under investigation. Moreover, the set of CVs should also describe any slow degree of freedom of the process using a minimum number of CVs. If one of the slow degrees of freedom is not considered, the dynamics along this direction is not accelerated, with immediate consequences for the accuracy of the estimated energy profile. Lack of any relevant degree of freedom will result in failure of the metadynamics simulations, and in inaccurate estimates of the free energy. Although a larger set of CVs might seem to be a solution to resolve the problem, the computational costs associated increase exponentially with the number of CVs, and redefining the entire set of CVs might be more advisable.

Metadynamics and Replica Methods

In practice, one usually deals with a suboptimal set of CVs to avoid an unreasonably large set. Under these circumstances, the equilibrium of neglected degrees of freedom that are still coupled to the reaction mechanism cannot be safely assumed. In these cases, several approaches have been exploited to accelerate relaxation along slow degrees of freedom. Parallel tempering metadynamics [32] and bias-exchange metadynamics (BE-META) [33] are two strategies that have been proposed to optimize the sampling of complex configurational spaces, such as those in ion channels. Both techniques avoid the exponential increase of the computational cost associated with the increase in the number of CVs by simulating different replicas of the system.

In parallel tempering metadynamics, multiple replicas of the system are simulated at different temperatures, and a metadynamics run is performed for each replica. Exchanges of configurations between replicas at adjacent temperatures are attempted at fixed intervals, using a Metropolis scheme that takes into account the different temperatures and biasing potentials of the replicas. The replicas at higher temperatures cross energy barriers more easily, which accelerates sampling of transition events along degrees of freedom that are not explicitly included in the set of CVs. Similarly, in BE-META, multiple replicas of the same system are employed and sampling is optimized by exchanging configurations among these replicas. However, in contrast to parallel tempering, in BE-META all the replicas are simulated at the same temperature but each

replica accelerates sampling along a different set of CVs. At fixed time intervals, exchanges of configurations between two randomly selected replicas are attempted, and the move is accepted or rejected with a probability that depends on the biasing potentials. Each metadynamics run accelerates sampling along its own set of CVs, and the exchange of configurations optimizes sampling of the degrees of freedom accelerated by the other replicas in the set. By using this strategy, it is possible to explore the space of CVs with high dimensionality more efficiently than in classical metadynamics simulations. Indeed, the computational cost of metadynamics simulations increases exponentially with the number of CVs. In BE-META simulation, each replica is usually accelerated along a 1-dimensional CV, and sampling in this low-dimensional space is extremely efficient. At the same time, the exchange of configurations between replicas, which are accelerated along different CVs, improves sampling of the high-dimensional space.

Application of metadynamics to the simulations of permeation in ion channels

The analysis of conduction events in ion channels requires accurate sampling of ion movements across the channel. Thus, a natural choice for the CVs in this case is the displacement of the ions along the pore axis. Figure 2 illustrates a representative simulation system of an ion channel inserted in a model cell membrane. A common strategy is to describe the movement of each individual ion by one CV. In ion channels where permeation is characterized by multi-ion conduction events, like in the case for example of potassium and sodium channels, the set of CVs needs to include any ion that takes part in conduction events. Figure 3 illustrates the established mechanisms of conduction of K^+ and Na^+ ions in potassium and sodium channels respectively reported from computational studies of the type describe in this review. In this section, application of metadynamics to study ion conduction will be compared with Umbrella Sampling (US) simulations.

US is likely the most popular method for calculating free energy profiles for conduction events in ion channels. The main practical difference between metadynamics and US is that in US the region of the configurational space sampled by the simulations is under strict manual control. Indeed, in order to estimate an energy profile by US, it is necessary to simulate a set of independent trajectories (windows), where each trajectory samples a

predefined region of the configurational space. As a consequence, an initial atomic configuration of the system needs to be defined for each independent atomic trajectory in this approach. This is a critical step because it is crucial that each window of an US simulation provides a representative sampling of the equilibrium ensemble in that particular region of the configurational space. The definition of the initial atomic configurations for the different windows might appear to be a rather trivial task. Indeed, if the CVs describe ion movements along the channel axis, the initial configurations could be defined by manually allocating ions to positions along the pore. However, this strategy might work efficiently only if the movement of the ion along the channel axis is the rate-limiting step in conduction; i.e. once these movements are accelerated, the other degrees of freedom of the system equilibrate rapidly. This condition is not always satisfied. In many ion channels, the number and the type of atoms forming the coordination shell of the permeating ions exert an important control on the mechanism of conduction. For instance, hydration/dehydration of an ion at the entrance of a channel is a common rate-limiting step in conduction. These processes could be accelerated by a CV that accounts for the number of water molecules in the first hydration shell of the ion. This strategy was used to accelerate sampling of ion movements in the ClC chloride channel in one of the first studies of permeation using metadynamics [34]. The set of CVs used to accelerate sampling of conduction in the ClC channel included the movement of ions along the channel axis, the coordination number of a glutamate residue in a constricted site along the pore, the distance between this glutamate residue and an interacting arginine residue, and the number of coordinating water molecules. It is not practical to manually assign initial configurations along this complicated set of CVs, and the consequence is that under similar conditions, the initial configurations for US simulations are usually defined by a preliminary exploration of the configurational space with other techniques, e.g. steered MD simulations. Instead, in metadynamics a single initial configuration of the system is required which simplifies the set-up of the simulation protocol and facilitates the use of any CV. In addition, the explored region of the CVs is predefined in US, while in metadynamics the system evolves naturally along the minimum energy pathways. This is an important feature over other techniques used to calculate free energy profiles as it guarantees an efficient use of computational resources.

We will use the bacterial Na^+ channels as a relevant example to illustrate the differences between metadynamics and US in the analysis of conduction. The pore of Na^+ channels presents several Na^+ binding sites (Figure 3). It has been observed in simulations that in some of these binding sites, a hydrated Na^+ ion is aligned with the central axis of the channel, while in alternative positions, the cation loses some of the water molecules in its coordination shell and it directly interacts with residues of the protein lining the pore [35].Equally this has been observed in simulations of non-selective TRPV channels [36]. Under these circumstances, in order to define accurately the position of an ion in the channel, two variables are required; one is the displacement along the channel axis and the other is the radial distance from this axis. As ion conduction in Na^+-channels involves at least two Na^+ ions, an energetic description of ion movements requires a 4-dimensional energy profile. As metadynamics simulation explored only the relevant regions in the 4-dimensional space of the CVs, only a 180-ns trajectory was enough to estimate the energy profile [37]. In contrast, it would have been extremely difficult to obtain similar efficiency with US simulations because the region sampled needs to be manually defined.

The shortcomings of US described earlier might be circumvented by the self-learning adaptive US method [38]. In this approach, a restricted region of the configurational space is first explored by manual definition of the simulation windows like in classical US. Then, the free energy in this region is estimated and new windows are created in the surrounding of the previous ones. Only windows in regions with energy below a pre-defined threshold are selected for an expansion step, and the procedure is iterated until convergence is achieved (i.e. it is not possible to create new windows within the pre-defined energy threshold). As windows are added in the proximity of low-energy regions, the system is forced to explore low-energy pathways, as in metadynamics. The higher control over the sampling space imposed in US simulations compared to metadynamics has also important positive consequences. The most important one is that in practice, higher accuracy of the estimated energy profiles is easier to achieve in US compared to metadynamics simulations. This feature is well illustrated in studies of conduction in K^+ channels [39].

The selectivity filter of K^+-channels is formed by a five residue sequence, TVGYG, termed the signature sequence, within the P loop of each subunit (Figure 3). Under

physiological K^+ concentrations, the selectivity filter residues arrange to form rings of carbonyl oxygen atoms directed toward the center of the pore axis. Four adjoining sites are defined, namely S1-S4. Dehydrated K^+ ions bind in these cage-like sites. Further binding sites for 'partially' hydrated K^+ ions have been also identified at the extracellular entrance (S_0, S_{EXT}) and within the central cavity (S_C) of the channel. Conduction of K^+ ions through the selectivity filter might proceed by different routes [40]. In the first mechanism of conduction that was atomistically described using the free energy perturbation technique, transitions of ions at S1, S3 and in the intracellular cavity of the channel, and ions in S0, S2, and S4 were proposed [41]. Subsequently, US simulations identified two alternative routes for this transition [42]. Along the minimum energy pathway, the ion in the intracellular cavity first approaches S4, and subsequently, ions in S3 and S1 move to S2 and S0. Alternatively, displacement of ions in S1 and S3 precedes the upward movement of the ion in the cavity. Metadynamics simulations performed to study this process were in qualitative agreement with the results from US simulations, although some of the alternative pathways were not identified probably as a consequence of limited sampling [43]. However, a longer trajectory does not guarantee a better agreement between US and metadynamics results. It is possible that in longer metadynamics runs, the biasing potential forces the system to explore configurations different from the ones sampled in US because for example ions might exist the selectivity filter, causing dramatic changes in the conformation of the selectivity filter, or 'extra' ions to those that we are tracking might arrive perturbing the free energy profile. These events might be prevented by the application of harmonic potentials that restraints the range of values explored by the CVs. In practice, this reduces the advantages of metadynamics over US. WTmetaD simulations are likely to be able to alleviate converge issues in metadynamics simulations. However, there does not seem to be any study illustrating the use of this technique in the investigation of conduction in ion channels in the literature.

At present, the most common strategy to overcome convergence issues of metadynamics simulations is to adopt a two-steps strategy [34, 44, 45]. Metadynamics simulations are used to efficiently sample permeation events, and to identify the minimum energy pathway for conduction. Subsequently, US simulations are used to get an accurate

estimate of the free energy profile along this pathway. This strategy unites the advantages of metadynamics (efficient sampling of complex configurational space and the possibility to use any type of CVs) with the ones of US (faster convergence of the energy profiles).

The features of the US algorithm make the estimate of stable free energy profiles easy. The negative side of this characteristic of US is that problems associated with a wrong choice of the CVs are not easy to appreciate. In contrast in metadynamics, if a wrong set of CVs has been chosen, the convergence of the biasing potential is not achieved. If a relevant slow degree of freedom is not included in the set of CVs, the biasing potential is likely to exhibit hysteresis, as configurations belonging to the same energy basin in the space of the CVs might actually be separated by high energy-barriers. The presence of hysteresis will reveal the problem, and analyses of the atomic trajectories might suggest possible solutions. Instead, in US simulations, each region of the configurational space is explored by a set of windows. The starting configurations for these windows are usually correlated among each other, and consequently it is extremely rare to sample movements along degrees of freedom orthogonal to the CVs included in the analysis. As an example, consider a hypothetic ion channel where ionic movements are described by two CVs: the displacement along the axis of the pore and the radial distance from the axis. Instead, the ion channel is studied using enhanced sampling techniques using a single CV: the displacement along the axis. In other words, energy barriers for ion movements exist both in the radial direction and along the axis, but only movements along the axis are accelerated in the simulations. In metadynamics, the permeating ion will exit and re-enter the pore, and hysteresis will become apparent when the ion enters the channel from different routes. In US simulations, the ion is restricted inside the channel, and as energy barriers exist in the radial direction, it will not explore alternative configurations. The consequence is that the wrong choice of the CV is more likely to be unnoticed in US simulations, leading to an estimate of energy profile that is only apparently converged to the free energy of the atomic system.

A common problem in the definition of the CVs for the analysis of conduction events is the number of ions to consider. Since the set of CVs is defined in advance, the number of ions that participate in conduction events is postulated. The first immediate consequence of this strategy is that the number of ions considered in free energy calculations might not

correspond to the number of ions that participate in reality. In this case, the energy profile is expected to be characterized by high-energy barriers, as the number of ions analyzed is not optimal for conduction. High-energy barriers were present in the energy profile of a single Na^+ ion crossing the bacterial Na^+ channel NaVAb [35, 46], which suggested that more than one Na^+ ion participates in conduction in this channel. Indeed, conduction in bacterial Na^+-channels was described as a process involving at least two or three ions [10, 35, 37, 46]. In atomistic MD simulations of ion conduction, the number of ions that participate in permeation does not need to be defined in advance and conduction mechanisms with different number of ions might be sampled during the simulation time. In contrast, when ion conduction is analyzed by free energy profiles along a predefined set of CVs, the number of ions is usually a parameter that needs to be set. Setting a priori the number of permeating ions restricts the region of the configurational space that is analyzed, and it might render an inaccurate estimate of the energy profile. For instance, if conduction mechanisms with two or three ions coexist, and the energy profile describes only conduction events with two ions, this energy profile is not fully representative of the distribution of ions in the channel. The coexistence of conduction mechanisms where different numbers of ions are involved at different times is not a peculiarity of Na^+ channels. In K^+-channels, conduction was initially studied using a three-ion process [42], but on the basis of free energy calculations using four ions, alternative conduction mechanisms were found energetically possible [40], as recently confirmed by direct MD simulations [47]. In principle, it is possible to estimate energy profiles with a variable number of ions inside the channel by sampling the higher dimensional space (conduction events with two ions are included in a 3-dimensional space). In practice, because of the computational cost associated with sampling a high dimensional space, this strategy is rarely adopted. Recently, we proposed an alternative approach based on BE-META simulations [48]. Preliminary tests on a toy model of a bacterial Na^+-channel illustrated that sampling conduction events with different number and type of ions inside the channel is feasible. This strategy might be useful to analyze conduction and selectivity in ion channels in conditions that more closely resemble the experimental ones.

In a recent study, by combining electrophysiology, X-ray crystallography and BE-META simulations, Laio and co-workers found that the pore region of cyclic nucleotide-gated

(CNG) channels exhibits a dynamic structure that underlies the coupling between gating, permeation and the poor ionic selectivity of CNG channels [49]. They also speculate about the structural basis for the distinctive regulation of K^+ and CNG channels. CNG channels, despite sharing a significant homology with K^+-channels, do not discriminate among monovalent alkali cations and are permeable also to several organic cations. Inspired by the electrophysiological experiments, the dipole moment component of the selectivity filter perpendicular to the bilayer plane was selected as a CV. Experiments also showed that the side chain of residue Glu66 in the selectivity filter assumes a variety of conformations. Therefore, to account for the long-range movement of the glutamate side chains, the four distances between the carboxylate and carbonyl groups of Glu66 on opposite monomers within the tetramer were also selected as CVs. The coordination number of the ions with the two oxygens of the carboxylic group of Glu66 was the third CV selected as well as the coordination pairs of the native contacts between carbonyl oxygens of Glu66 residues and the hydroxyl group of the nearby Tyr55 and between carboxylic oxygens of Glu66 residues and the hydroxyl group of Thr60. As a result, BE-META predicted that Glu66 and the prolines in the outer vestibule undergo large fluctuations, which are modulated by the ionic species and the voltage.

Application of metadynamics to model gating in ion channels

All known ion channels can be regulated by an external stimulus that can be, for instance, a ligand binding, a transmembrane potential, pressure, or even temperature. Gating is the property of ion channels that determines if the channel is open, and so ions can pass through, or if it is closed and thus, the flow of ions is restricted. The gate is the region of the channel that hampers the permeant ions to diffuse through the pore in the closed state. In relation to gating, activation refers to the conformational changes of the main intracellular gate of ion channels involving large scale steric occlusion of the pore. In contrast, inactivation refers to the conformational changes at the selectivity filter toward a nonconductive state. The selectivity filter is a constricted structural element of the protein that is able to discriminate between ionic species. In K^+ channels, when the selectivity filter of ion channels acts as a gate switching between conducting and non-conducting states the process is known as C-type inactivation. Inactivation occurs on a variety of timescales from tens of milliseconds to seconds. Activation is a stimulus-dependent

process, while inactivation is a spontaneous one. The two processes are thought to be dynamically coupled [50]. MD simulations revealed atomic details about the coupling between gating and inaction [51], and about the microscopic events leading to state transitions in the selectivity filter [52]. Understanding what triggers gating, what happens during gating, and how it depends on the structure of the channel are questions of paramount importance to understand how these proteins work.

In the context of this review, insight into the microscopic factors responsible for C-type inactivation in K^+-channels were reported after an extensive computational study on the KirBac channel, using metadynamics. These computations supported the existence of a physical gate or constriction in the selectivity filter of K^+-channels [53]. The computations identified a new selectivity filter structure, which was proposed to be associated with C-type inactivation. During the inactivation process, the four peptide-chains that comprise the filter adopt an unusual structure in which their dihedral angles alternate between left- and right-handed Ramachandran angles, which also justifies the need for conservation of glycine in the K^+-channel selectivity filter. During the gating process, a cooperative conformational change involves K^+ ions and residues from the selectivity filter. Nevertheless, the conformational rearrangement of two out of the four opposite chains is asynchronous and caused by a transient asymmetric movement of the two ions that occupy the selectivity filter. The global symmetry of the open structure changes from four- to twofold, as the structure of the selectivity filter evolves along through three stages. All four subunits are involved, and the interactions between subunits are mediated through the ion-conducting pore and through direct side chain or backbone interactions. These results were consistent with a model whereby the conformational changes during the inactivation proceeds by a multistep process and where the presence or absence of K^+ ions in particular position in the selectivity filter are a key factor in such a mechanism. Subsequently, a series of crystal structures of the potassium channel KcsA trapped in various degrees of gate opening and ion occupancy were reported [50]. These structures provided direct evidence to the type and extent of the structural changes associated with C-type inactivation. Future work in this area would likely concentrate on clarifying the mechanism of recovery from inactivation in order to determine the pathways connecting the different functional states of the channel. Large-

scale conformational transitions represent both a challenge and an opportunity for computer simulation. At present, application of path-like CVs [54] and metadynamics to determine the kinetics and thermodynamics associated with gating without the need of prohibitively long trajectories is possibly one of the most attractive and accessible frameworks.

Conclusions

The widespread physiological implications of ion channels emanate from their modulation of electrical signals across the cellular membrane. Establishing the mechanisms of ion permeation, selectivity and gating in ion channels is at the forefront of biomedical sciences and is fundamental for understanding the relation between ion channel structure and function. For many years, molecular dynamics simulations have played a fundamental role in framing these relationships. However, all-atom simulations suffer from several limitations. In particular, two issues of utmost importance need to be considered and addressed. First, the accuracy of the force fields employed is well-known to be far from optimal [55]. Second, the sampling efficiency required to explore several conformational states and to compare their free energies accurately. An applied external electric field can be used to model the ionic conductance over a broad range of applied voltages. The numbers of ions that traverse the channel per unit time are then counted and an estimate of the conductivity is obtained. However, this approach is computationally very expensive, and more efficient alternatives are essential. In this review, the focus has been on how to study ion channels using techniques to accelerate sampling of rare events. Recently, enhanced sampling methods have been successfully applied to investigate ion channel permeation and gating. In particular, one of the many available algorithms to overcome the sampling problem is the so-called metadynamics. Metadynamics is a family of enhanced sampling techniques aimed at enhancing the rare events and reconstructing the underlying free energy landscape as a function of a set of order parameters. Studies of ion conduction with this algorithm have predicted permeation pathways and the related binding free energy profiles. One major challenge in addition to the choice of the CVs is the accurate description of the conformational complexity during conduction. Studies addressing efficient sampling of ion channel conformations during permeation have been reviewed.

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Figures

Figure 1. Free energy estimate from a metadynamics simulation. The free energy profile of a Na^+ ion moving along the selectivity filter of a bacterial Na^+ channel is shown. The process was described by a 1-dimensional collective variable: the displacement of a Na^+ ion (green sphere) along the axis of the channel. Another Na^+ ion (yellow sphere) was present in the simulation domain, thus, the profile corresponds to the energy experienced by the first ion in the presence of the second one. The black line corresponds to the energy profile once the simulation has converged. The green continuous line corresponds to the sum of Gaussian energy terms in a generic snapshot of the metadynamics trajectory, i.e. the biasing potential. This biasing potential modifies the energy acting on the ion (dashed green line equal to the sum of free energy and biasing potential), raising the energy of local energy minima, and forcing the system to explore new configurations.

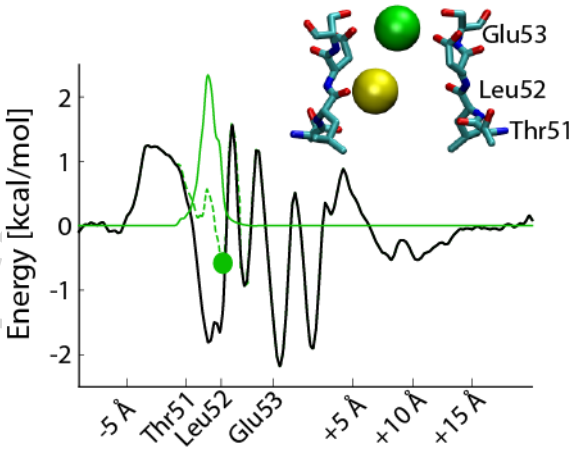

Figure 2. Model simulation system. Cartoon of the overall architecture of the transmembrane domain of an ion channel inserted in a model lipid bilayer. Only two chains in red and blue are shown for clarity. Ions are represented as green and white spheres.

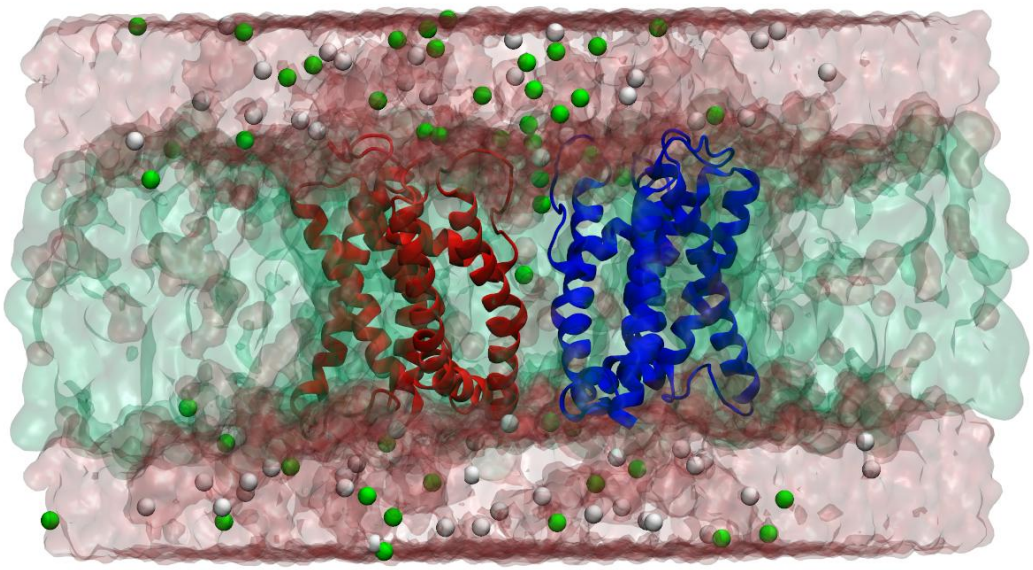

Figure 3. Proposed mechanisms of ion conduction in K^+ and Na^+ channels. (a) Chains of alternating K^+ ions and/or ions and water molecules cross the selectivity filter. Colored spheres represent individual K^+ ions. (b) Selectivity filter residues are represented in licorice and Na^+ ions are orange and green spheres. In both cases, (a) and (b) approach of an ion from the central cavity prompts simultaneous ion movement throughout the selectivity filter, resulting in the exit of one ion that rapidly diffuses into the extracellular solution.